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Reactivation of Human Herpesvirus 6 by Infection of Human Herpesvirus 7

Keiko Tanaka-Taya,[1,2]* Toshio Kondo,[3] Naoko Nakagawa,[4] Reiko Inagi,[2] Hiroko Miyoshi,[1] Tomimasa Sunagawa,[1] Shintaro Okada,[1] and Koichi Yamanishi[2]

[1]Department of Developmental Medicine (Pediatrics) D-5, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan
[2]Department of Microbiology C-1, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan
[3]Kondo Pediatric Clinic, Toyonaka City, Osaka, Japan
[4]Research Institute of Osaka Prefecture, Osaka City, Osaka, Japan

We have attempted to reactivate human herpesvirus 6 (HHV-6) by infection with HHV-7 using childhood exanthem subitum patients in vitro. Peripheral blood mononuclear cells (PBMCs) were collected from children who had a history of exanthem subitum(ES) by HHV-6 and were infected by human herpesvirus 7 (HHV-7) in vitro. The antigen positive rate to HHV-6 started to increase 7 days after the infection and reached a maximum by Day 15 using an immunofluorescence antibody test. The copy number of HHV-6 DNA also increased in the samples in 10 days after infection in vitro. No antigen or increase in DNA was detected in PBMCs, that were mock-infected or infected with supernatant of stock virus after ultracentrifugation, suggesting that an infection by HHV-7 is necessary to reactivate HHV-6. In the paired sera samples during the acute and the convalescent phases of ES, seven to ten bands, that were specific for HHV-6, were recognized in samples from the acute phase, and at least 5 dominant polypeptides were found more intensively after HHV-7 infection. ***J. Med. Virol. 60:284–289, 2000.***

KEY WORDS: HHV-6; HHV-7; in vitro reactivation; antibody titer; PCR; exanthem subitum

INTRODUCTION

Human herpesvirus 6 (HHV-6) was first isolated from patients with lymphoproliferative disorders [Salahuddin et al., 1986]. In 1990, Frenkel et al., isolated another lymphotropic herpesvirus from a healthy adult and named it human herpesvirus 7 (HHV-7) [Frenkel et al., 1990]. These two herpesviruses have similar characteristics such as T cell tropism, but they can be clearly distinguished by immunological and molecular biological techniques [Frenkel et al., 1990; Berneman et al., 1992]. The main clinical manifestation of the primary HHV-6 and -7 infections was febrile illness [Pruksananonda et al., 1992; Hall et al., 1994; Torigoe et al., 1995] and exanthem subitum(ES) [Yamanishi et al., 1988; Tanaka et al., 1994]. Seroepidemiological investigations show that most children have antibody to HHV-6 before 2 years of age [Okuno et al., 1989; Briggs et al., 1990]. In contrast, HHV-7 infects children somewhat later in childhood [Wyatt et al., 1991; Yoshikawa et al., 1993; Tanaka-Taya et al., 1996]. HHV-6 latently infects after the primary infection in human monocytes/macrophages [Kondo et al., 1991] and persistently infects in salivary glands [Fox et al., 1990]. Under immunosuppressive conditions, such as AIDS or transplant recipients, they reactivate and cause various complications [Okuno et al., 1990; Carrigan et al., 1991; Yoshikawa et al., 1991; Cone et al., 1993; Drobyski et al., 1993; Yalcin et al., 1994; Drobyski et al., 1994].

When HHV-7 infection occurs in patients who already have antibodies to HHV-6, the antibody titer to HHV-6 increases in the patients [Tanaka-Taya et al., 1994; Ueda et al., 1994], and in vitro HHV-7 provides a transacting function mediating HHV-6 reactivation from latency [Frenkel and Roffman, 1996; Katsafanas et al., 1996]. In this paper, we describe that the HHV-6 is reactivated by HHV-7 infection in vitro, and we also discuss the mechanism of the reactivation of HHV-6 by HHV-7.

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*Correspondence to: Dr. Keiko Tanaka-Taya, Dept. of Developmental Medicine (Pediatrics) D-5, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan.

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MATERIALS AND METHODS

Patients

Five patients (Patients 1–5) who were clinically diagnosed as having exanthem subitum (ES) were studied using serological tests. Eight other patients (Patients 6–13), ages 1–3 with a history of ES by HHV-6 was studied by collection of their peripheral blood mononuclear cells (PBMCs). Informed consent for blood sampling was obtained from all the children's parents.

Antibody Test

The indirect immunofluorescence test for serum antibody to HHV-6 and -7 was carried out as described previously [Asada et al., 1989; Okuno et al., 1989; Tanaka et al., 1994; Tanaka-Taya et al., 1996]. The results were read without the knowledge of the clinical phase of the patients. Serological cross reactivity with HHV-6 was excluded because there were patients who were positive for HHV-7 only or HHV-6 only, as reported previously [Tanaka et al., 1994].

Immunoprecipitation of Viral Proteins

Immunoprecipitation procedures and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were used as described previously with some modifications [Okuno et al., 1992]. Briefly, to analyze the polypeptides recognized by the serum from the children during the acute and the convalescent phases of ES by HHV-7, umbilical cord blood lymphocytes (CBLs) were infected with HHV-6 (HST strain) and cultured for a few days. When 30–50% of the cells showed cytopathic effect (CPE), they were radio-labeled with [^{35}S]methionine (40–50 mCi/ml) for 16 hours in methionine-free Eagle's MEM (EMEM) with 3% fetal calf serum. The cells were collected by centrifugation at 3,000 rpm at room temperature and cell-pellets were solubilized with RIPA buffer [0.01M Tris HCl pH 7.4, 0.15M NaCl, 1% sodium deoxycholate, 1% Triton-X, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The radiolabeled antigens were mixed with serum, and using Protein G-Sepharose (Pharmacia) precipitated immune complexes. The immunoprecipitated antigens were eluted from the sepharose using sample buffer containing 2-mercaptoethanol and were analyzed by SDS-PAGE.

Procedure for Reactivation of HHV-6 by HHV-7 Infection In Vitro

The PBMCs collected from the patients during the convalescent phase of ES were cultured as described previously [Yamanishi et al., 1988] in vitro for 3 days. The cells were then divided into 3 parts: one for HHV-7 infection, one for mock-infection and one for infection with the supernatant of HHV-7 stock. One portion was infected with HHV-7 (7-KHR strain) at a multiplicity of infection (MOI) of 1 by centrifugation at 2,500 rpm for 45 minutes; the second portion was infected with the supernatant of HHV-7 culture medium after ultracentrifugation at 25,000 rpm for 1 hour to precipitate the virus; and the last portion was infected with the medium (mock-infected). The cultured cells were collected every few days, and stained with monoclonal antibodies to HHV-6 (OHV-1) [Okuno et al., 1992] and HHV-7 (KR-4) by immunofluorescence test. To investigate the cross-reactivity between HHV-6 and HHV-7, we stained the HHV-6 (HST strain) and HHV-7 (7-KHR strain) using the both monoclonal antibodies to HHV-6 (OHV-1) and HHV-7 (KR-4).

Detection of HHV-6 DNA

To detect HHV-6 DNA in the cultures, 10^5 cultured cells from the 3 parts (HHV-7 infected cells, HHV-7 culture medium infected cells and mock infected cells) were collected in 10 days after the HHV-7 infection and treated with K-buffer as described previously [Kondo et al., 1990]. Then the DNA was amplified by polymerase chain reaction (PCR) and followed by a southern blot hybridization as described previously [Yamamoto et al., 1994; Yalcin et al., 1994; Tanaka-Taya et al., 1996]. The DNA thermal cycler condition was a 1-minute denaturation step at 94°C , a 2-minute annealing step at 62°C , and an elongation step of 3-, 4-, or 5-minute at 72°C for 10 cycles. The amplification reaction was carried out using a DNA thermal cycler (Perkin-Elmer/Cetus). The detection of the DNA by PCR was attempted at least twice for each sample, and the results were the same on each occasion. The amplified samples were visualized on a 2% agarose gel electrophoresis stained with ethidium bromide. To confirm the results of PCR amplification, hybridization technique with biotin conjugated oligonucleotide probes was carried out as previously described [Yalcin et al., 1994; Tanaka-Taya et al., 1996]. The sequences of specific oligodeoxy-nucleotide probes for HHV-6 A and B were: 5′-GAACTCCATCAGCGGCCTCCAG-3′ (HHV-6A) and 5′-TAAATCCATTACTGGCCTTGAA-3′ (HHV-6B).

Specificity and Sensitivity of HHV-6 Probes

The specificity of the PCR was evaluated by hybridization of DNA extracted from cells infected with human herpesviruses: herpes simplex virus types 1 and 2, varicella-zoster virus, human cytomegalovirus, Epstein-Barr virus, HHV-7. Amplification was not detected even by the southern blot hybridization (data not shown).

Quantification of the PCR Reaction

We have attempted to apply DNA amplification by the following method for semiquantitative PCR. We always used 1×10^5 PBMCs as the template for PCR. To determine sensitivity we used control plasmids of known concentration derived from HHV-6 DNA immediate early gene with DNA from HHV-6 (–) 10^5 cells.

TABLE I. Clinical Features and Antibody Responses to HHV-6 and -7

Patient number features	Phase	Antibody titer to HHV-7	Antibody titer to HHV-6	Clinical
1	Acute	<10	<10	Exanthem subitum
	Convalescent	160	<10	
2	Acute	<10	5120	Exanthem subitum
	Convalescent	160	20480	
3	Acute	<10	320	Exanthem subitum
	Convalescent	640	2560	
4	Acute	<10	2560	Exanthem subitum
	Convalescent	80	5120	
5	Acute	<10	1280	Exanthem subitum
	Convalescent	320	2560	

RESULTS

Antibody Responses to HHV-6 and -7

All the 5 patients, who were clinically diagnosed as having ES, were detected to have no antibody to HHV-7 during the acute phase of ES, but were detected to have antibody to HHV-7 during the convalescent phase, suggesting a primary HHV-7 infection (Table I). Four patients (2–5) were detected to have antibody to HHV-6 during the acute phase, and the antibody titers to HHV-6 were boosted in these patients during the convalescent phase of ES. Both the HHV-6 and the HHV-7 were isolated from peripheral blood mononuclear cells of Patient 2 during the acute phase of ES for confirmation, using monoclonal antibodies specific to HHV-6 or HHV-7 (data not shown).

Immunoprecipitation Test

The paired sera samples from 2 patients, obtained during the acute and the convalescent phases of ES, were used for the immunoprecipitation test. Seven to ten bands, that were specific for HHV-6, were recognized in samples from the acute phase (Fig.1, lanes 2 and 6), and at least 5 dominant polypeptides with molecular weight of 130 kDa, 110 kDa, 82 kDa, 62 kDa and 48 kDa, were found more intensively after HHV-7 infection.

Reactivation of HHV-6 by HHV-7 Infection

HHV-6 (HST strain) was not stained by the monoclonal antibody to HHV-7 (KR-4), and HHV-7 (7-KHR strain) was not stained by the monoclonal antibody to HHV-6 (OHV-1), respectively. It shows these monoclonal antibodies did not document cross-reactivity.

The PBMCs collected from other 8 children (6–13) who already had antibody to HHV-6 but not to HHV-7 were cultured for 3 days and then infected with HHV-7. Cells were collected every few days after the HHV-7 infection and stained with monoclonal antibodies to HHV-6 (OHV-1) and HHV-7 (KR-4). HHV-7 antigen was detected in 3–4 days postinfection. Whereas HHV-6 antigen was not detected in 3–4 days postinfection, the antigen positive cells were detected in 7 days postinfection and those increased until 14 days postinfection. The numbers of antigen positive cells, however, decreased thereafter because of destruction of cells caused by HHV-7 replication (Fig. 2). No specific antigen to HHV-6 was detected in samples, that were mock-infected cells.

Quantification of the PCR

The sensitivity of single PCR with ethidium bromide staining and southern blot hybridization followed by single PCR were $>10^3$ and 10^2–10^3 genome equivalents, respectively. (data not shown).

Detection of HHV-6 DNA from 10^5 Cultured Cells After HHV-7 Infection by PCR

To confirm the reactivation of HHV-6 by HHV-7 infection, the semiquantitative PCR was attempted by staining with ethidium bromide and then southern blot hybridization. The PBMCs obtained from 3 patients (6–8) were cultured for 10 days after HHV-7 infection and 10^5 cells were collected for the detection of HHV-6 DNA by PCR. In Patient 6, $>10^3$ copies of HHV-6 DNA could be detected out of the 10^5 PBMCs infected with HHV-7 (6c), but HHV-6 DNA could not be detected in the uninfected control cells (6a) or the sample infected with supernatant of HHV-7 (6b). In Patient 7 and 8, 10^2–10^3 copies of HHV-6 DNA could be detected out of the 10^5 PBMCs infected with HHV-7 (7c and 8c), but HHV-6 DNA could not be detected in the uninfected control cells (7a and 8a) or the sample infected with supernatant of HHV-7 (8b). For Patient 7 faint hybridization shows in the sample infected with supernatant of HHV-7. Approximately 10^2 copies of HHV-6 DNA could be detected in them (7b). (Fig. 3).

DISCUSSION

HHV-6 and -7, that belong to a betaherpesvirus subfamily, are the causative agents for exanthem subitum [Yamanishi et al., 1988; Tanaka et al., 1994]. After a primary infection such as ES, they infect latently and reactivate in hosts when the patients have immunosuppressive conditions such as organ transplant, AIDS, or other viral infection [Okuno et al., 1990; Carrigan et al., 1991; Yoshikawa et al., 1991; Drobyski et al., 1993; Cone et al., 1993; Yalcin et al., 1994; Drobyski et al., 1994]. HHV-6 infects infants early in life, but HHV-7 infects later [Wyatt et al., 1991; Yoshikawa et al., 1993; Tanaka-Taya et al., 1996]. Therefore, it is speculated that almost all children have antibody to HHV-6 in a normal condition before the primary infec-

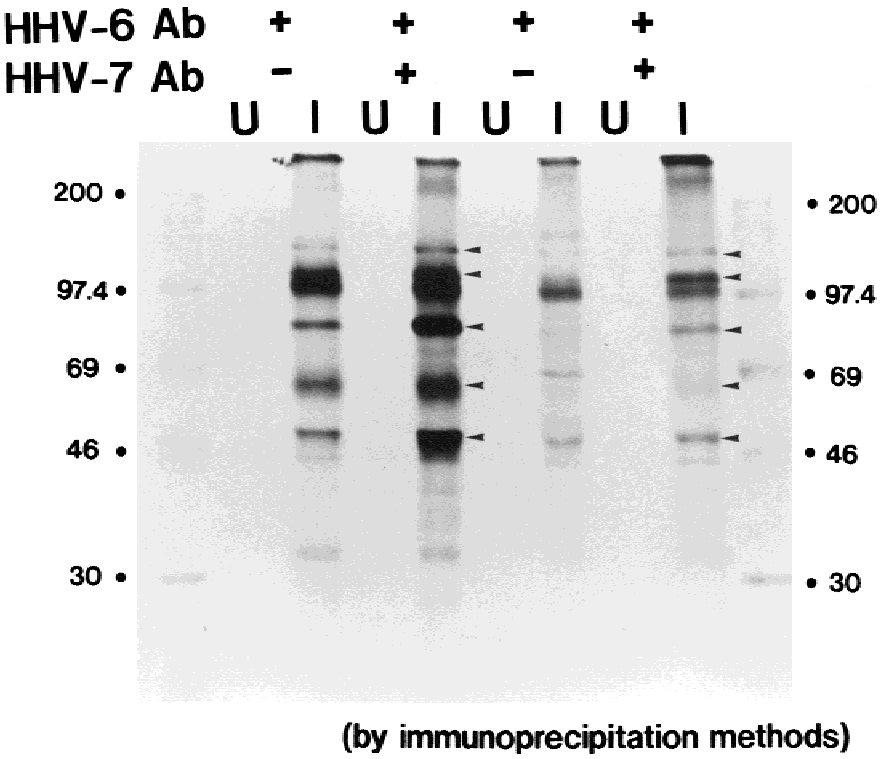

Fig. 1. Reactivation of HHV-6 by HHV-7 with immunoprecipitation methods. Ab: antibody; +: positive; −: negative; U: uninfected cord blood mononuclear cells; I: HHV-6 infected cord blood mononuclear cells; ◀: HHV-6 specific proteins; ·: molecular marker.

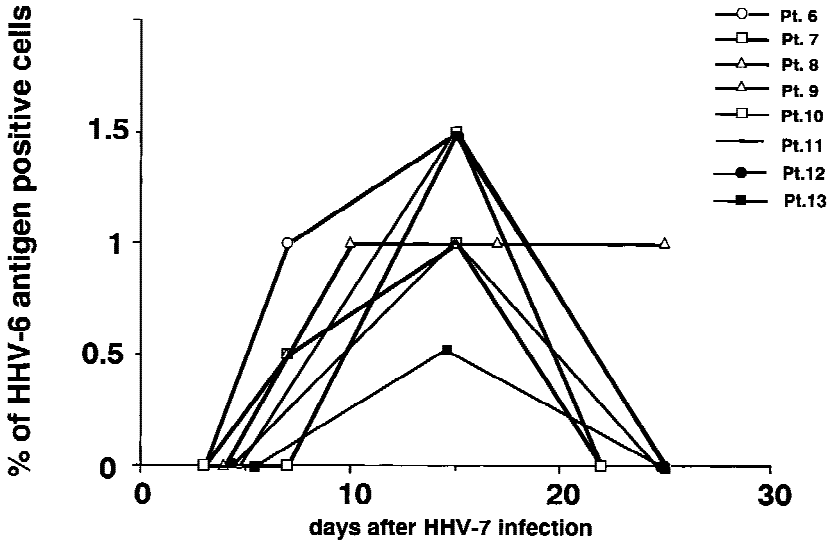

Fig. 2. Reactivation of HHV-6 by HHV-7 with IFA methods. Detection of HHV-6 antigen positive cells by monoclonal antibody to HHV-6 (OHV-3) after HHV-7 infection in vitro.

tion of HHV-7. Interestingly, antibody titer to HHV-6 is boosted during the convalescent phase of HHV-7 infection [Tanaka et al., 1994; Ueda et al., 1994], and this was confirmed in this study by serological examination. These observations suggest the following hypothesis: 1) Because HHV-6 and -7 are antigenically related [Wyatt et al., 1991], the cross-reactive antibodies can be boosted; 2) Some genes of HHV-7 may transactivate those of HHV-6 and may stimulate HHV-6 replication. In fact, it was reported that HHV-6 activates the Epstein-Barr virus (EBV) replication cycle [Flamand et al., 1993]; 3) HHV-6 can be reactivated in vivo during the primary HHV-7 infection by an indirect mechanism such as suppression of immunity in patients during the acute phase of HHV-7 infection, or induction of several cytokines, that may stimulate HHV-6 replication. It was reported during the early convalescent stage of measles that HHV-6 was reactivated and was isolated from the PBMCs [Suga et al., 1992].

To analyze these possibilities, an immunoprecipitation test was firstly attempted. As shown in Figure 1, not only cross-reactive antigens between HHV-6 and -7 but also other antigens to HHV-6 were boosted by HHV-7 infection, suggesting that HHV-6 may replicate

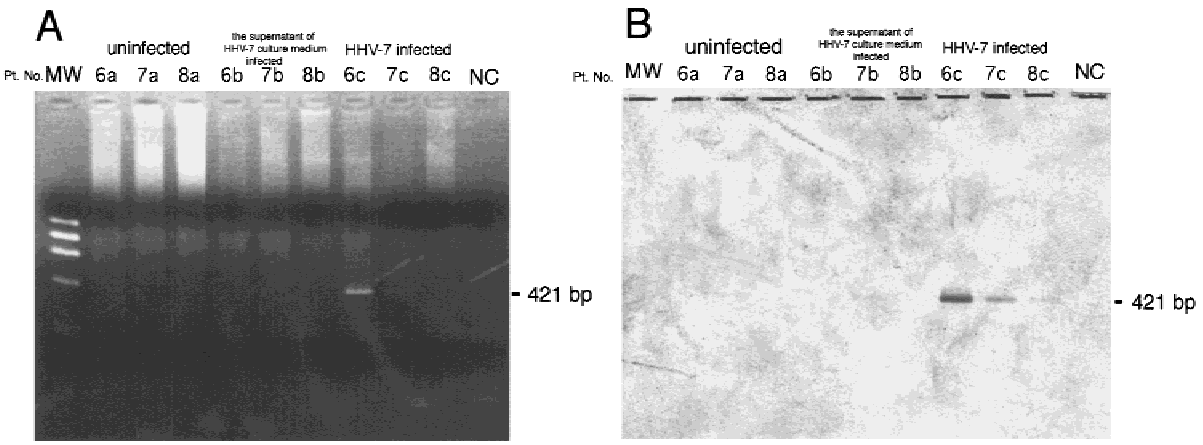

Fig. 3. Reactivation of HHV-6 by HHV-7 with PCR and southern blot hybridization. (**A**) Uninfected control. (**B**) The supernatant of HHV-7 culture medium infected 10^5PBMCs.(**C**) HHV-7 infected 10^5PBMCs. MW: molecular weight; NC : negative control; Pt. No.: Patient number 6–8.

in patients with a primary HHV-7 infection. As described in Results, we were able to isolate HHV-6 during the primary infection of HHV-7 from the sample of Patient 2. It was difficult, however, to culture HHV-6 continuously without the contamination with HHV-7. Therefore, we tried to detect antigens and DNA of HHV-6 in vitro by infection with HHV-7 to mononuclear cells from patients who were already infected with HHV-6. Antigens to HHV-6 were not detected in the mock-infected and in the early stage (until 7 days) after the HHV-7 infection in vitro, but the number of HHV-6 positive cells increased thereafter (Fig. 2). This result was confirmed by PCR (Fig. 3). These results suggested that HHV-7 infection helps HHV-6 replication in cells, that were latently infected, with HHV-6 or HHV-7 replication proteins could replicate the HHV-6 genome given their close relation. It was not clear, however, whether this effect is direct or indirect. It was reported that HHV-7 can provide a transacting function mediating HHV-6 reactivation from latency by passage of HHV-7 infected PBMCs from healthy individuals [Frenkel and Roffman, 1996; Katsafanas et al., 1996]. In our study HHV-6 can be isolated from latency and a high number of HHV-6 copies can be detected after HHV-7 infection without passage. These results show that latent infection with HHV-6 can be reactivated easier from PBMCs of children than of healthy adults. On the other hand, HHV-7 may stimulate some cytokines in cells as reported in HHV-6 [Flamand et al., 1991]. In a sample infected with supernatant of HHV-7, HHV-6 DNA could be detected lower than in the sample infected HHV-7. These results suggest that HHV-7 is needed directly for the reactivation of HHV-6; however, additional and more detailed studies are needed to determine the mechanism of HHV-6 reactivation during the HHV-7 infection. Furthermore, it is not clear yet whether HHV-6 replicates in organs, like skin, during the primary HHV-7 infection and causes some clinical symptoms. For clarification, pathological studies are necessary in the future.

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